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Note

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Application of High-Throughput Competition Experiments in the Development of Aspartate-Directed Site-Selective Modification of Tyrosine Residues in Peptides

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ABSTRACT: Herein we report a Cu-catalyzed, site-selective functionalization of peptides that employs an aspartic acid (Asp) as a native directing motif, which directs the site of O-arylation at a proximal tyrosine (Tyr) residue. Through a series of competition studies conducted in high-throughput reaction arrays, effective conditions were identified that gave high selectivity for the proximal Tyr in Asp-directed Tyr-modification. Good levels of site-selectivity were achieved in the O-arylation at a proximal Tyr residue in a number of cases, including a peptide-small molecule hybrid.

Modified peptides can exhibit potent bioactivity as drugs and serve as highly selective chemical probes in drug development.[1] As their utility continues to increase with advances in drug discovery,[2] there is a need to generate diverse analogues of these molecules, as well as hybrid peptide-small molecule structures, which could exhibit improved pharmacological properties. The vast growth in this area has resulted in a high demand for methods that enable late-stage peptide modification.[3] One of the key difficulties encountered in developing bioconjugative chemistries that target a single amino acid residue is achieving site-selectivity in the presence of multiple copies of the target amino acid.[4] The use of a non-native coordinating motifs, which can direct a catalyst or reagent to the proximal reactive site, has been a successful approach to address this challenge.[5] However, this multi-step process is synthetically inefficient and invariably leads to increased waste since it requires installation and removal of the directing groups. As a complementary approach, site-selective peptide modification has emerged as a field of study.[6] Presented below is a contribution predicated on using a naturally occurring amino acid as a directing group in the context of increasingly complex peptidic substrates.

Carboxylate functionality has been used as a common directing group for transition-metal catalysis,[7] often accelerating the rate of reactions or aiding in accomplishing enantioselective functionalizations (Figure 1a). To achieve site-selectivity in the context of peptide modification, we imagined that carboxylate-containing residues, such as an Asp, would function as a suitable metal-binding motif that directs functionalization to proximate residues.

While reports that modify nucleophilic residues such as lysine[8] and cysteine[9] are relatively abundant, additions to the lexicon of methods that target tyrosine[10] residues could be particularly welcome. Recently, some of us reported the selective O-functionalization of phenols in small molecules via copper-catalyzed cross-coupling reaction,[11] which we felt could

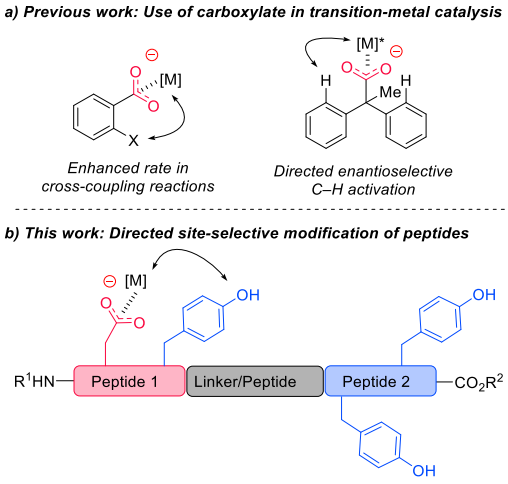


Figure 1. a) Selected examples of a carboxylates as a directing group in transition-metal catalysis. b) Our proposed use of carboxylate group in site-selective peptide functionalization.

be well suited for such a transformation. In those reports, an aryl bromide containing an ortho-trifluoroacetamide developed by Ma and co-workers allowed us to employ relatively mild conditions for the cross-coupling reaction.[12] Herein, we

describe a directed approach to Cu-catalyzed, site-selective O-arylation of peptides that bears multiple Tyr residues (Figure 1b).

The site-selective modification of peptides presents inherent challenges due to their chemical and structural diversity. Additionally, reaction analysis in such systems can be difficult due to formation of multiple constitutional isomers, which often have ambiguous NMR and mass data. As such, there is a need for a systematic, rapid protocol to assess peptidic motifs of interest. Rather than choosing a single representative peptide sequence for this complex challenge, we designed a series of intermolecular competition experiments between multiple peptide substrates to study the kinetic significance of the proposed Asp-metal interactions (Figure 2). Such an approach offers 1) a high level of modularity, allowing for the rapid comparison of different motifs, 2) the easy synthesis of authentic products for identification, and 3) the easy separation and identification of products through simple LC-MS techniques. Furthermore, we envisioned utilizing high-throughput experimentation (HTE) techniques to enable an efficient exploration of large arrays of catalysts and reaction conditions.[13] We imagined that such a protocol could be applied in the modification of various complex peptides, even proteins.

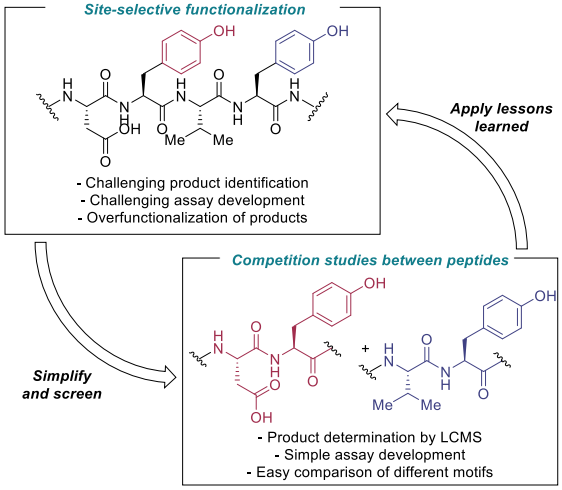


Figure 2. Our proposed HTE protocol towards site-selective modification of peptides.

We began our studies by performing competition experiments between four dipeptides containing different Boc-XX-Tyr-OMe dyad motifs. These initial efforts identified that in-house developed guanidinylated ligand **L1** was capable of selectively arylating di-peptides containing Asp or Glu residues, while no preference was shown for di-peptides containing a methylated Asp(OMe) residue (see SI for full details). With these initial results in mind, we decided to examine tetrapeptides **1a-d**, each with a unique *i* + *1* residue and a Tyr at the *i* + *2* position which we felt would better represent Tyr residues imbedded within longer sequences. In addition to evaluating the desired Asp-Tyr dimeric sequence (**1a**), we

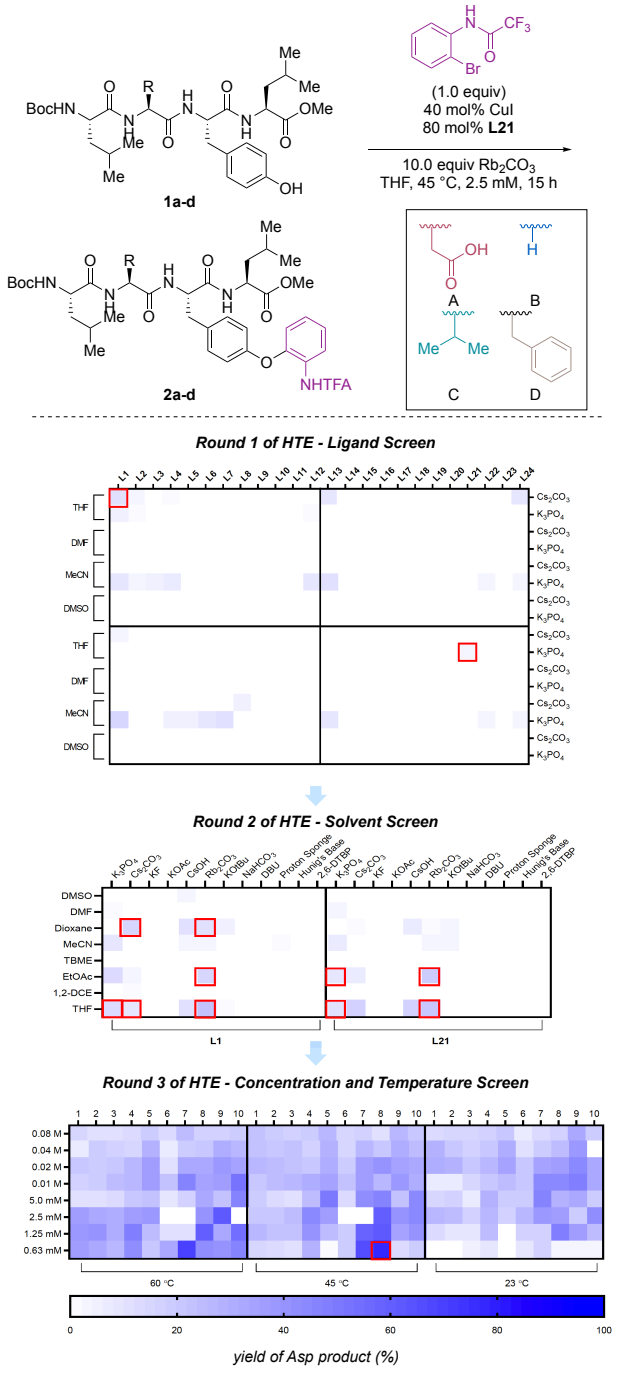


Figure 3. Optimization of competition experiments via high-throughput experimentation (HTE)

elected to examine tetrapeptides containing glycine (**1b**), valine (**1c**) and phenylalanine (**1d**) in order to assess a variety of steric environments without the addition of heteroatoms.

Upon examining 24 ligands in combination with common literature solvents, bases, and copper sources on a μmol scale in a 96-well array (See SI, section 4.3 for full details), several promising ligands were identified (Figure 3, round 1, red boxes). Gratifyingly, ligand **L1** provided the desired product **2a** in 23% yield, which consisted of 55% of all arylated products. We were also intrigued by the oxalate ligand **L21** developed by the Ma group,[14] which afforded the desired peptide **2a** as the exclusive product albeit not in the highest yield. These ligands

were selected for further examination in the second round of HTE wherein we more broadly examined solvents and bases (See SI, section 4.5 for full details). Rb_2CO_3 provided a considerable increase in selectivity compared to using more commonly employed Cs_2CO_3. Of all the conditions examined, 10 "optimal" combinations of bases (Cs_2CO_3, Rb_2CO_3, and K_3PO_4) and solvents (EtOAc, THF, and dioxane) were selected for final optimization of temperature and concentration (Figure 3, round 2, red boxes). Through this third round of HTE, we observed that generally more dilute conditions promoted selective formation of **2a**. The efficacy of dilute conditions suggests that selectivity is more likely a function of each individual peptide sequence rather than the behavior of an aggregated species. To our delight, the optimal HTE conditions provided 93% total yield of all four arylated peptides, with 81% selectivity for **2a**. While this high selectivity was reproducible on a mmol scale, the overall yield of the reaction diminished to 51% (Figure 4). Re-examining high performing reaction conditions identified during HTE, we found that using THF at 60 °C with 10.0 equivalents of base proved to be superior on a mmol scale. In fact, while the reaction provides an overall yield of 65%, **2a** consists 91% of the arylated products.

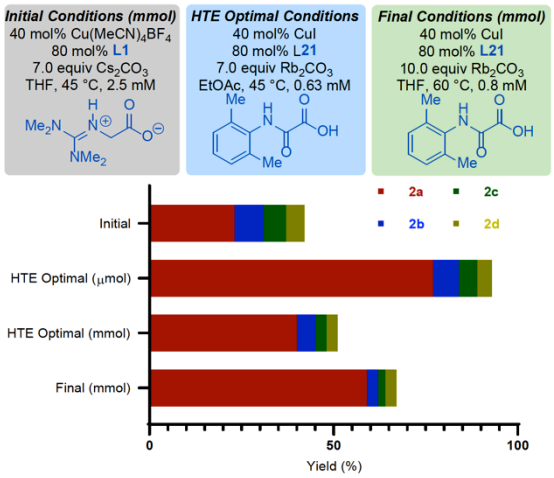
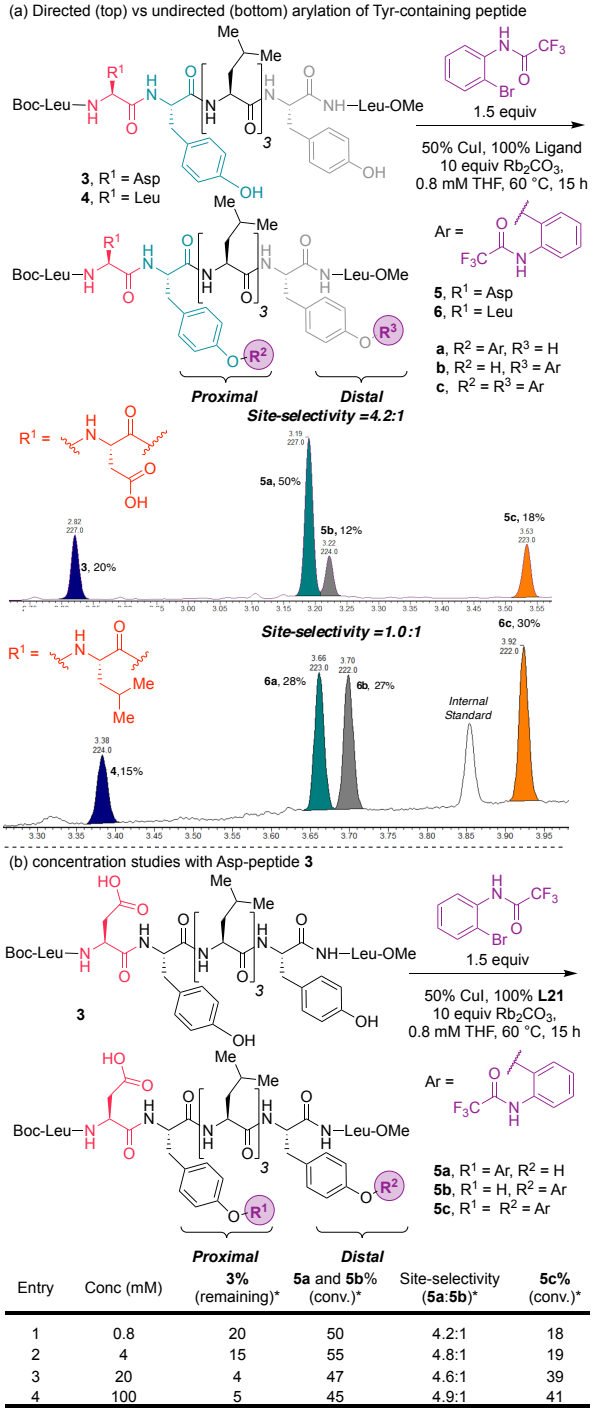


Figure 4. A comparison of initial (μmol scale), HTE (μmol and mmol scale), and final (mmol scale) conditions.

With the finalized conditions in hand, we then began to study site-selective cross-coupling reactions with peptides containing multiple Tyr residues. In agreement with our HTE competition studies, treatment of a peptide that bears an Asp (**3**) with the Cu/**L21** catalyst and aryl bromide resulted in a good level of the proximal site-selectivity (4.2:1, Figure 5a, top) for the Cu-catalyzed O-arylation. In stark contrast, peptide **4**, which lacks an Asp residue, demonstrated no preference for reactivity at either Tyr residue (1.0:1, Figure 5a, bottom), consistent with Asp directed selectivity. While we suspect that selectivity is conferred via carboxylate coordination to a Cu species, the exact mechanism is currently unknown. Further studies in which the reaction concentration was increased showed that the site-selectivity remains mostly unaffected, suggesting minimal impact from potential aggregation effects (Figure 5b). The incremental gain in the selectivity may be due to the enhanced overall reactivity that resulted in conversion of the minor product **5b** to the bis-coupled product **5c**.



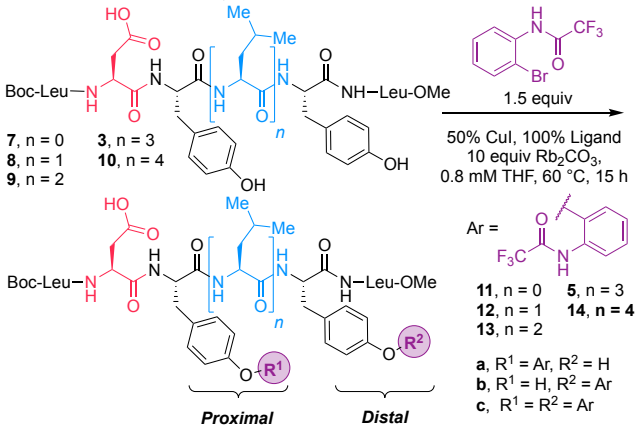
Entry	Conc (mM)	3% (remaining)*	5a and 5b% (conv.)*	Site-selectivity (5a:5b)*	5c% (conv.)*
1	0.8	20	50	4.2:1	18
2	4	15	55	4.8:1	19
3	20	4	47	4.6:1	39
4	100	5	45	4.9:1	41

Figure 5. Experiments for probing Asp residue as a directing group in site-selective arylation of peptides. Relative product ratios were determined using LC-MS UV% analysis. a) (top) Site-selective arylation of Tyr in presence of a directing residue (Asp). Authentic compounds were prepared for the identification of each arylated product. (bottom) Non-directing residue (Leu) leads to non-selective arylation. b) Site-selectivity is mostly unaffected by concentration.

We then decided to probe the effect of distance between the Tyr residues on site-selectivity. Greater distance between the Tyr residues led to higher selectivity, affording 5.1:1 site-selectivity when four Leu units separated the proximal and distal Tyr residues (Table 1, Entry 5). On the other hand,

essentially unselective O-arylation is observed with substrates where the Tyr residues are in close proximity to one another (Entries 1 and 2). We speculate that the directing ability of Asp residue may extend over more than one amino acid residue due to structural flexibility of the peptides.

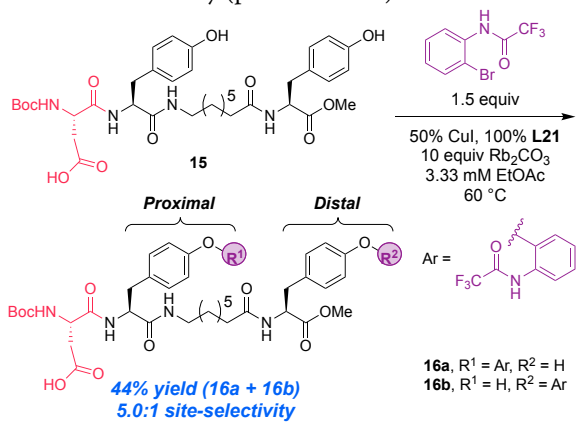
Table 1. Studies on the effects on the level of site-selectivity and the distance between Tyr residues



Entry	# of spacers (n)	SM% (remaining)*	Mono (**a** + **b**)% (conv.)*	Site-selectivity (**a**:**b**)*	**c**% (conv.)*
1	0	12	57	1.1:1	31
2	1	12	55	1.2:1	33
3	2	13	53	1.9:1	34
4	3	20	62	4.2:1	18
5	4	30	62	5.1:1	7

*Relative product ratios were determined using LC-MS UV% analysis.
Ligand used is 2-(2,6-dimethylphenylamino)-2-oxoacetic acid

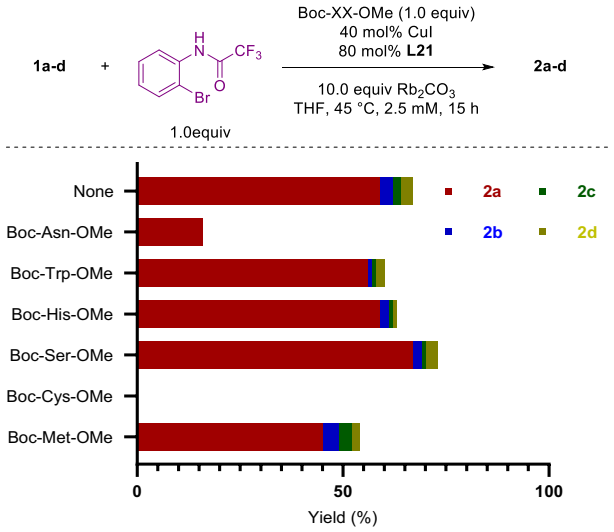
Inspired by the abundance of hybrid peptides with applications in medicine and biotechnology,[15] we also examined hybrid peptide **15** with Asp-Tyr and distal Tyr motif (Scheme 1). The number of methylene (-CH_2) units in **5** were chosen such that the number of atoms between the two Tyr residues are equal in compound **3** and **15**. Under slightly modified conditions, we obtained mono-arylated products in 44% yield with 5.0:1 site-selectivity (proximal:distal).



Scheme 1. Site-selective arylation of peptide-hybrid **15**

While our studies demonstrated that Asp residues could be used to bias reactivity between two Tyr residues, we wondered if site-selectivity could also be achieved in the presence of other nucleophilic amino acid residues that might undergo competitive arylation. In order to rapidly assess the influence of other residues, we conducted further competition studies with protected monomer additives, similar to approaches taken by Glorius and co-workers.[16] We were pleased to find that the selectivity largely remained high for the Asp-directed product **2a** in most cases. For example, nucleophilic residues such as Trp, His and Ser had minimal impact on both

Table 2. Robustness screen using protected amino acid monomers.



selectivity and reactivity of the reaction. However, the presence of Asn and Cys proved to drastically inhibit reactivity. While a full assessment of substrates dense with functionality is beyond the scope of this study, these preliminary results suggest that the presented strategy could perform well in more complex environments.

Invariably, the complexity of peptidic substrates presents a plethora of potential directing groups for any metal-catalyzed derivatization. However, the studies presented above reveal that there exists a substantial opportunity to achieve site-selectivity in a predictable manner. Competition experiments emerged as a useful strategy for identifying one such opportunity with Asp-functionalized peptides. HTE provided a particular advantage for rapidly identifying a set of conditions that could be applied to test the operation of the directing effects in more complex settings. That the directing effects held up in substrates armed with an Asp residue proximal to a Tyr, in contrast to control substrates lacking the Asp residue, bodes well for further exploration of sequence-dependent, site-selective cross-coupling chemistry.

General Information. Room temperature is defined as 21–23 °C. All reagents were purchased from commercial suppliers and used without further purification, unless otherwise noted. Acetonitrile (MeCN), dichloromethane (DCM), *N,N*-dimethylformamide (DMF) and tetrahydrofuran (THF) were obtained from a Seca Solvent System by GlassContour, in which the solvent was dried over alumina and dispensed under an atmosphere of Ar. All other solvents were purchased from

commercial suppliers and used without further purification, unless otherwise noted. Routine ^{1}H NMR spectra were recorded on Agilent 400, 500, or 600 MHz spectrometers at ambient temperature unless otherwise stated. All NMR solvents were purchased from Cambridge Isotope Laboratories and used without further purification. Chloroform-*d*, dichloromethane-d_2 and deuterium oxide-d_2 were stored at ambient temperature, and methanol-d_4 and dimethylsulfoxide-d_6 ampoules were used immediately after opening. Spectra were processed using MestReNova 10.0.1 using the automatic phasing and polynomial baseline correction capabilities. Splitting was determined using the automatic multiplet analysis function with manual intervention as necessary. Spectral data are reported as follows: chemical shift (multiplicity [singlet (s), broad singlet (brs), doublet (d), triplet (t), quartet (q), pentet (p), multiplet (m), doublet of doublets (dd), doublet of doublet of doublets (ddd), doublet of triplet of doublets (dtd), doublet of doublet of doublet of doublets (dddd), doublet of triplets (dt), triplet of doublets (td), etc.], coupling constant, integration). Chemical shifts are reported in ppm (δ), and coupling constants are reported in Hz. 1H Resonances are referenced to solvent residual peaks for $CDCl_3$ (7.26 ppm), CD_2Cl_2 (5.32 ppm) and CD_3OD (3.31 ppm).[17] Routine ^{13}C NMR spectra were recorded on an Agilent 600 MHz spectrometer with protons fully decoupled. ^{13}C Resonances are reported in ppm relative to solvent residual peaks for $CDCl_3$ (77.2 ppm), CD_2Cl_2 (53.8 ppm) or CD_3OD (49.0 ppm).[17] Note: Small deviations in chemical shifts may be observed depending on the concentration of NMR samples. Infrared spectra were recorded on a Nicolet 6700 ATR/FT-IR spectrometer, and ν_{max} are partially reported in cm^{-1}. Samples for high-resolution mass spectrometry were submitted to the Mass Spectrometry Laboratory at the University of Illinois at Urbana-Champaign, in which data was acquired on a Waters Synapt G2-Si instrument equipped with an ESI detector, or were obtained on a Yale Waters ZQ 4000 Single Quad LCMS. Analytical thin-layer chromatography was performed using 60 Å Silica Gel F254 pre-coated plates (0.25 mm thickness). TLC plates were visualized by irradiation with a UV lamp. R_f values are reported for non-peptidic compounds. Normal-phase flash chromatography was performed using a Biotage Isolera One purification system equipped with a 10, 25, or 50 g SNAP Ultra (HP Sphere, 25 μm silica) cartridge and an appropriate EtOAc/Hex or MeOH/DCM linear gradient in the mobile phase. Reversed-phase column chromatography was performed using a Biotage Isolera One purification system equipped with a 60 or 120 g SNAP-C18 column and an appropriate MeOH/H_2O or MeCN/H_2O linear gradient in the mobile phase.

Previously Synthesized Compounds. Guanidinylated ligand **L1**[11] and oxalamide ligand **L21**[18] were synthesized following the previously reported procedures.

General solution-phase peptide coupling procedure. Solution-phase peptide synthesis was performed using the Boc protecting group strategy.[11] Representative synthesis is as follows: to a flask equipped with a magnetic stir bar was added H-Tyr(OBn)-OMe•HCl (1.00 g, 3.11 mmol, 1.00 equiv) and DCM (15.50 mL, 0.2 M). Boc-Asp(α-OBn)-OH (1.21 g, 3.73 mmol, 1.00 equiv), HOBT•H_2O (0.57 g, 3.73 mmol, 1.20 equiv), EDC•HCl (0.72 g, 3.73 mmol, 1.20 equiv). DIPEA (1.30 mL, 7.46 mmol, 2.40 equiv) were added to the stirring solution. Reaction mixture was allowed to stir at rt, and the reaction was monitored using LC-MS (usually complete conversion within 12 h). Upon complete conversion, the solution was poured into a separatory funnel and diluted with DCM. The organic layer was washed with 10% (w/v) aqueous citric acid. The organic layer was separated and subsequently washed with saturated aqueous $NaHCO_3$ and brine. The organic layer was then dried over anhydrous Na_2SO_4, filtered and concentrated in vacuo to provide the dimeric peptide as a white foam. Crude peptide dimer was then treated with 4 M HCl in 1,4-dioxane (3.90 mL, 15.5 mmol, 5.00 equiv). The solution was allowed to stir for at rt, and the reaction was monitored by LC-MS (usually complete conversion within 1 h). Excess HCl was removed under an intense stream of N_2 for 1 h which was vented into a saturated solution of $NaHCO_3$ (aq). The remaining solvent was removed in vacuo to provide peptide dimer Boc-Asp(α-OBn)-Tyr(OBn)-OMe, which was dried thoroughly under reduced pressure before being carried forward to the next coupling step.

Procedure 1: Hydrogenolysis of Peptides. All peptides were prepared using O-Benzyl protected tyrosine residues and were deprotected under hydrogenolysis conditions. A flask equipped with a magnetic stir bar was added Boc-Leu-Asp(OBn)-Tyr(OBn)-Leu-OMe (3.100 g, 3.80 mmol, 1.00 equiv) 10 wt % Pd/C (0.402 g, 0.38 mmol, 0.10 equiv) evacuated with N_2 for 15 min, after which a balloon of hydrogen was added. MeOH (19.0 mL, 0.2 M) was slowly added, and the reaction was left to stir at room temperature overnight (15 h). The mixture was filtered through Celite® and concentrated in vacuo to give a clear oil. Crude material was purified by reversed-phase chromatography.

Boc-Leu-Asp-Tyr-Leu-OMe (1a) was synthesized following the solution-phase peptide coupling procedure followed by Procedure 1. The crude material was then purified by reversed-phase chromatography (20→100% MeOH/H_2O) to provide the desired product as a white solid (38% yield). ^{1}H NMR (600 MHz, CD_3OD) δ 7.04 (d, *J* = 8.0 Hz, 2H), 6.69 (d, *J* = 8.1 Hz, 2H), 4.63 (t, *J* = 6.4 Hz, 1H), 4.51 (t, *J* = 6.7 Hz, 1H), 4.44 (dd, *J* = 9.8, 4.9 Hz, 1H), 4.04 (t, *J* = 7.4, 6.3 Hz, 1H), 3.67 (s, 3H), 3.07 (dd, *J* = 14.1, 5.7 Hz, 1H), 2.84 (m, 2H), 2.71 (dd, *J* = 17.1, 6.5 Hz, 1H), 1.66 (m, 3H), 1.57 (m, 1H), 1.47 (t, *J* = 7.3 Hz, 2H), 1.44 (s, 9H), 0.98 – 0.84 (m, 12H); ^{13}C{^{1}H} NMR (151 MHz, CD_3OD) δ 175.7, 174.2, 173.2, 172.5, 158.1, 157.2, 131.3, 128.9, 116.2, 80.8, 56.3, 54.7, 52.7, 52.2, 51.3, 41.8, 41.4, 37.6, 36.3, 28.8, 25.9, 25.7, 23.5, 23.4, 21.9, 21.8; IR (FT-ATR, cm^{-1}, neat) ν_{max} 2960, 1648, 1516, 1390, 1367, 1226, 1160, 1022, 829. HRMS m/z $[M+H]^+$ calcd for $C_{31}H_{48}N_4O_{10}$ 637.3443, found 637.3433 (ES+).

Boc-Leu-Gly-Tyr-Leu-OMe (1b) was synthesized following the Solution-phase peptide coupling procedure followed by Procedure 1. The crude material was then purified by reversed-phase chromatography (20 → 100% MeOH/H_2O) to provide the desired product as a white solid (28% yield). ^{1}H NMR (600 MHz, CD_3OD) δ 7.04 (d, *J* = 8.1 Hz, 2H), 6.68 (d, *J* = 8.0 Hz,

2H), 4.57 (dd, *J* = 8.5, 5.7 Hz, 1H), 4.44 (dd, *J* = 9.8, 5.1 Hz, 1H), 4.04 (dd, *J* = 9.9, 5.3 Hz, 1H), 3.87 (d, *J* = 16.8 Hz, 1H), 3.74 (d, J = 16.5 Hz, 1H), 3.68 (s, 3H), 3.07 (dd, *J* = 14.1, 5.7 Hz, 1H), 2.83 (dd, *J* = 14.1, 8.6 Hz, 1H), 1.72 – 1.49 (m, 6H), 1.44 (s, 9H), 1.01 – 0.83 (m, 12H); $^{13}C\{^{1}H\}$ NMR (151 MHz, CD_3OD) δ 176.3, 174.2, 173.5, 171.2, 158.1, 157.3, 131.3, 128.9, 116.2, 80.7, 56.1, 54.8, 52.7, 52.2, 43.5, 41.9, 41.5, 38.0, 28.8, 25.9, 25.8, 23.5, 23.3, 21.9, 9; IR (FT-ATR, cm^{-1}, neat) ν_{max} 3296, 2957, 1644, 1515, 1439, 1367, 1234, 1161, 1022, 827; HRMS m/z $[M+H]^+$ calcd for $C_{29}H_{46}N_4O_8$ 579.3388, found 579.3389 (ES+).

Boc-Leu-Val-Tyr-Leu-OMe (1c) was synthesized following the Solution-phase peptide coupling procedure followed by Procedure 1. The crude material was then purified by reversed-phase chromatography (20 → 100% $MeOH/H_2O$) to provide the desired product as a white solid (42% yield). ^1H NMR (600 MHz, CD_3OD) δ 7.02 (d, *J* = 8.0 Hz, 2H), 6.77 – 6.57 (m, 2H), 4.56 (t, *J* = 7.4 Hz, 1H), 4.42 (dd, *J* = 10.0, 5.1 Hz, 1H), 4.15 (d, *J* = 6.9 Hz, 1H), 4.06 (t, *J* = 7.7 Hz, 1H), 3.63 (s, 3H), 2.99 (dd, *J* = 14.0, 6.6 Hz, 1H), 2.79 (dd, *J* = 14.0, 8.0 Hz, 1H), 1.99 (h, *J* = 6.9 Hz, 1H), 1.70 – 1.50 (m, 4H), 1.48 (t, J = 7.4 Hz, 2H), 1.42 (s, 9H), 0.93 (d, *J* = 6.6 Hz, 3H), 0.90 (dt, *J* = 6.8, 1.7 Hz, 6H), 0.86 (t, *J* = 5.4 Hz, 9H); $^{13}C\{^{1}H\}$ NMR (151 MHz, CD_3OD) δ 175.6, 174.1, 173.3, 173.0, 157.2, 131.3, 128.7, 116.2, 80.7, 59.7, 55.9, 54.6, 52.7, 52.0, 41.7, 41.6, 38.0, 32.4, 28.7, 25.9, 25.7, 23.5, 23.4, 21.9, 21.8, 19.7, 18.5; IR (FT-ATR, cm-1, neat) ν_{max} 3277, 2957, 1639, 1514, 1367, 1234, 1167, 1046, 1020, 827, 663; HRMS m/z $[M+H]^+$ calcd for $C_{32}H_{52}N_4O_8$ 621.3858, found 621.3865.

Boc-Leu-Phe-Tyr-Leu-OMe (1d) was synthesized following the Solution-phase peptide coupling procedure followed by Procedure 1. The crude material was then purified by reversed-phase chromatography (20 →100% $MeOH/H_2O$) to provide the desired product as a white solid (51% yield). ^1H NMR (600 MHz, CD_3OD) δ 7.22 – 7.21 (m, 2H), 7.18 –7.16 (m, 3H), 7.02 (d, *J* = 8.0 Hz, 2H), 6.68 (d, *J* = 8.2 Hz, 2H), 4.58 (dd, *J* = 8.5, 5.5 Hz, 1H), 4.54 (t, *J* = 7.1 Hz, 1H), 4.44 (dd, *J* = 9.5, 5.6 Hz, 1H), 3.99 (dd, *J* = 9.3, 5.9 Hz, 1H), 3.65 (s, 3H), 3.06 (dd, *J* = 14.0, 5.5 Hz, 1H), 3.00 (dd, *J* = 14.0, 6.3 Hz, 1H), 2.87 (dd, *J* = 14.0, 8.4 Hz, 1H), 2.81 (dd, *J* = 14.0, 7.9 Hz, 1H), 1.69 – 1.54 (m, 4H), 1.42–1.31 (m, 12H), 1.38 – 1.31 (4H), 0.91 (td, J = 22.3, 20.7, 6.5, 1.2 Hz, 12H); $^{13}C\{^{1}H\}$ NMR (151 MHz, CD_3OD): δ 175.4, 174.2, 173.2, 172.8, 157.9, 157.3, 138.1, 131.4, 130.4, 129.4, 128.7, 127.7, 116.2, 80.7, 56.0, 55.5, 54.7, 52.7, 52.1, 42.0, 41.6, 38.8, 38.1, 28.8, 25.9, 25.8, 23.4, 23.3, 22.0, 21.9; IR (FT-ATR, cm^{-1}, neat) ν_{max} 3282, 2955, 1640, 1515, 1367, 1230, 1166, 1022, 829, 697; HRMS m/z $[M+H]^+$ calcd for $C_{36}H_{52}N_4O_8$ 669.3858, found 669.3857 (ES+).

Procedure 2: Procedures for competition experiment between tetrapeptides. To an oven dried 50 mL Schlenk flask equipped with a magnetic stir bar was added **1a** (0.020 mmol, 1.00 equiv), **1b** (0.020 mmol, 1.00 equiv), **1c** (0.020 mmol, 1.00 equiv), **1d** (0.020 mmol, 1.00 equiv) *N*-(2-bromophenyl)-2,2,2-trifluoroacetamide (0.0054 g, 0.020 mmol, 1.00 equiv), Cu source (0.008 mmol, 0.40 equiv), ligand (0.016 mmol, 0.80 equiv), and base (0.064 mmol, 3.20 equiv). The flask was sealed with a new rubber septum and further secured with Parafilm M®. The flask was placed under vacuum for 5 min and backfilled with N_2. This process was repeated two additional times. Solvent (32 mL, 2.5 mM) was added through the septum, and the mixture was allowed to stir for 15 h at 45 °C in an oil bath. The mixture was diluted with EtOAc and transferred to a separatory funnel. The organic layer washed with a solution of saturated NH_4Cl (aq). The organic layer was separated and the aqueous layer was extracted with EtOAc again. The organic layer was washed three times with brine. The combined organic layers were then dried over anhydrous Na_2SO_4, filtered, and concentrated in vacuo. The crude residue was then dissolved in 10 mL of DMSO. 1 mL of DMSO was then added which contained a known amount of di-*tert*-butylbiphenyl to serve as an internal standard (0.0536 g in 100 mL). An aliquot was then submitted for analysis by LC-MS.

Procedure 3: Synthesis of authentic products for monotyrosine containing compounds. To an oven dried 10-mL Schlenk flask equipped with a magnetic stir bar was added tyrosine-containing peptide (0.10 mmol, 1.00 equiv), *N*-(2-bromophenyl)-2,2,2-trifluoroacetamide (0.0300 g, 0.11 mmol, 1.10 equiv), $Cu(MeCN)_4BF_4$ (0.0064 g, 0.02 mmol, 0.20 equiv), *N*,*N*-dimethylglycine (0.0037 g, 0.02 mmol, 0.20 equiv), and Cs_2CO_3 (0.1300 g, 4.00 mmol, 4.00 equiv) which was flame dried under vacuum before use. The flask was sealed with a new rubber septum and further secured with Parafilm M®. The flask was placed under vacuum for 5 min and backfilled with N_2. This process was repeated two additional times. THF (2.5 mL, 0.04 M w.r.t. tyrosine-containing peptide) was added through the septum, and the mixture was allowed to stir for 15 h at 45 °C in an oil bath. Reaction mixture was diluted with EtOAc and transferred to a separatory funnel. The organic layer was washed with a solution of saturated NH_4Cl (aq). The organic layer was separated and the aqueous layer was extracted with EtOAc again. The organic layer was washed three times with brine. The combined organic layer was then dried over anhydrous Na_2SO_4, filtered, and concentrated in vacuo. The crude material was purified by reversed-phase chromatography.

Boc-Leu-Asp-Tyr(O-(2-(2,2,2-trifluoroacetamido)phenoxy)-Leu-OMe (2a) was synthesized following Procedure 3 from **1a**. The crude material was then purified by reversed-phase chromatography (20 →100% $MeOH/H_2O$) to provide the desired product as a white solid (43% yield). ^1H NMR (600 MHz, CD_3OD) δ 7.65 (dd, *J* = 8.0, 1.6 Hz, 1H), 7.27 – 7.23 (m, 3H), 7.15 (td, *J* = 7.7, 1.4 Hz, 1H), 6.96 – 6.94 (m, 3H), 4.63 (t, *J* = 6.3 Hz, 1H), 4.58 (dd, *J* = 8.2, 5.7 Hz, 1H), 4.44 (dd, *J* = 9.9, 4.9 Hz, 1H), 4.02 (t, *J* = 8.9, 1H), 3.65 (s, 3H), 3.16 (dd, *J* = 14.1, 5.4 Hz, 1H), 2.95 (dd, *J* = 14.1, 8.2 Hz, 1H), 2.80 (dd, *J* = 17.1, 6.3 Hz, 1H), 2.71 (dd, *J* = 17.1, 6.4 Hz, 1H), 1.72 – 1.61 (m, 3H), 1.61 – 1.52 (m, 1H), 1.52 – 1.45 (m, 2H), 1.42 (s, 9H), 0.96 – 0.84 (m, 12H); $^{13}C\{^{1}H\}$ NMR (151 MHz, CD_3OD) 175.8, 174.2, 173.0, 172.5, 158.2, 157.3 (d, *J* = 37.1 Hz), 156.5, 152.2, 134.2, 131.9, 129.3, 127.2, 124.4, 120.1, 119.6, 117.5 (d, *J* = 287.4 Hz), 80.8, 56.1, 54.8, 52.7, 52.2, 51.3, 41.8, 41.4, 37.8, 36.2, 28.8, 25.9, 25.8, 23.5, 23.4, 21.9, 21.8; ^{19}F NMR (376 MHz, CD_3OD) δ –77.98; IR (FT-ATR, cm^{-1}, neat) ν_{max} 2958, 1734, 1646, 1504, 1457,

1417, 1231, 1170; HRMS m/z $[M+H]^+$ calcd for $C_{39}H_{52}F_3N_5O_{11}$ 824.3688, found 824.3680 (ES+).

Boc-Leu-Gly-Tyr(O-(2-(2,2,2-trifluoroacetamido)phenoxy)-Leu-OMe (2b) was synthesized following Procedure 3 from **1b**. The crude material was then purified by reversed-phase chromatography (20 →100% MeOH/H_2O) to provide the desired product as a white solid (31%yield). ^{1}H NMR (600 MHz, CD_3OD) δ 7.62 (dd, *J* = 8.0, 2.2 Hz, 1H), 7.28 – 7.18 (m, 3H), 7.15 (dt, *J* = 7.6, 1.8 Hz, 1H), 6.96 – 6.90 (m, 3H), 4.62 (dd, *J* = 8.8, 5.7 Hz, 1H), 4.46 – 4.41 (m, 1H), 4.04 (dd, *J* = 9.6, 5.5 Hz, 1H), 3.87 (d, *J* = 16.6, 1H), 3.73 (d, *J* = 17.3 Hz, 1H), 3.68 (s, 3H), 3.17 (dd, *J* = 13.9, 5.5 Hz, 1H), 2.92 (dd, *J* = 13.8, 8.6 Hz, 1H), 1.72 – 1.50 (m, 6H), 1.44 (s, 9H), 0.96 – 0.88 (m, 12H); ^{13}C{^{1}H} NMR (151 MHz, CD_3OD): δ 176.3, 174.2, 173.4, 173.3, 171.3, 158.1, 157.3 (q, *J* = 37.2 Hz), 156.6, 152.1, 134.0, 131.9, 129.3, 127.3, 124.6, 119.9, 119.9, 117.5 (q, *J* = 287.3 Hz) 80.7, 55.9, 54.9, 52.7, 52.3, 52.2, 43.5, 41.9, 41.4, 38.1, 28.8, 25.8, 23.3, 21.9; ^{19}F NMR (376 MHz, CD_3OD) δ –76.98; IR (FT-ATR, cm^{-1}, neat) ν_{max} 2957, 1733, 1642, 1507, 1457, 1277, 1231, 1152, 760; HRMS m/z $[M+H]^+$ calcd for $C_{37}H_{50}F_3N_5O_9$ 766.3633, found 766.3629 (ES+).

Boc-Leu-Val-Tyr(O-(2-(2,2,2-trifluoroacetamido)phenoxy)-Leu-OMe (2c) was synthesized following Procedure 3 using **1c**. The crude material was then purified by reversed-phase chromatography (20 →100% MeOH/H_2O) to provide the desired product as a white solid (39%). ^{1}H NMR (600 MHz, CD_3OD) δ 7.66 (dd, J = 8.0, 1.6 Hz, 1H), 7.33 – 7.21 (m, 3H), 7.16 (td, *J* = 7.7, 1.4 Hz, 1H), 6.96 – 6.92 (m, 3H), 4.65 (q, *J* = 7.2 Hz, 1H), 4.47 – 4.43 (m, 1H), 4.19 (t, *J* = 7.2 Hz, 1H), 4.08 (dd, *J* = 9.0, 6.2 Hz, 1H), 3.66 (s, 3H), 3.12 (dd, *J* = 14.0, 6.7 Hz, 1H), 2.91 (dd, *J* = 14.0, 7.9 Hz, 1H), 2.02 (h, *J* = 6.8 Hz, 1H), 1.69 – 1.47 (m, 6H), 1.44 (s, 9H), 0.96 – 0.87 (m, 18H); ^{13}C{^{1}H} NMR (151 MHz, CD_3OD): δ 175.7, 174.2, 174.2, 173.1, 158.0, 157.3 (q, *J* = 37.0), 156.5, 152.2, 133.9, 131.9, 129.3, 127.2, 124.5, 120.1, 119.7, 117.5 (q, *J* = 286.7), 80.7, 59.8, 54.7, 52.7, 52.1, 52.0, 41.6, 38.1, 32.4, 28.7, 25.9, 25.7, 23.4, 21.8, 19.7, 18.5; ^{19}F NMR (376 MHz, CD_3OD) δ –76.99; IR (FT-ATR, cm^{-1}, neat) ν_{max} 1734, 1635, 1506, 1456, 1418, 1278, 1232, 1153; HRMS m/z $[M+H]^+$ calcd for $C_{40}H_{56}F_3N_5O_9$ 808.4103, found 808.4095 (ES+).

Boc-Leu-Phe-Tyr-(O-(2-(2,2,2-trifluoroacetamido)phenoxy)-Leu-OMe (2d) was synthesized following Procedure 3 using **1d**. The crude material was then purified by reversed-phase chromatography (20 →100% MeOH/H_2O) to provide the desired product as a white solid (43% yield). ^{1}H NMR (600 MHz, CD_3OD) δ 7.65 (dd, *J* = 7.9, 1.6 Hz, 1H), 7.23 (ddd, *J* = 7.4, 5.6, 1.7 Hz, 5H), 7.18 – 7.13 (m, 4H), 6.95 – 6.92 (m, 3H), 4.63 – 4.59 (m, 2H), 4.45 (dd, *J* = 9.4, 5.7 Hz, 1H), 4.00 (t, *J* = 8.0 Hz, 1H), 3.67 (s, 3H), 3.10 (ddd, *J* = 27.1, 14.0, 5.8 Hz, 2H), 2.95 – 2.87 (m, 2H), 1.71 – 1.54 (m, 4H), 1.42 (s, 9H), 1.38 – 1.32 (m, 2H), 0.95 (d, *J* = 6.6 Hz, 3H), 0.90 (dd, *J* = 13.7, 6.6 Hz, 6H), 0.86 (d, *J* = 6.6 Hz, 3H); ^{13}C{^{1}H} NMR (151 MHz, CD_3OD): δ 175.4, 174.2, 172.9, 172.9, 172.7, 157.9, 157.3 (q, *J* = 37.3 Hz), 156.5, 152.2, 138.1, 133.9, 132.0, 130.4, 129.4, 129.3, 127.7, 127.2, 124.5, 120.0, 119.7, 117.5 (q, *J* = 287.3 Hz), 80.7, 55.7, 55.5, 54.7, 52.7, 52.1, 42.0, 41.5, 38.8, 38.2, 28.8, 25.8, 23.4, 23.3, 22.0; ^{19}F NMR (470 MHz, CD_3OD) δ –76.96; IR (FT-ATR, cm^{-1}, neat) ν_{max} 3315, 2955, 1640, 1515, 1367, 1235, 1164, 1021; HRMS m/z $[M+H]^+$ calcd for $C_{44}H_{56}F_3N_5O_9$ 856.4103, found 856.4092 (ES+).

Solid-phase peptide synthesis. The SPPS via the Fmoc protecting group strategy was conducted using similar to previously reported procedures.[19] See SI for representative synthesis and LC-MS retention times of peptides along with eluent conditions.

Boc-Leu-Asp-Tyr-Leu-Leu-Leu-Tyr-Leu-OMe (3) was synthesized from Fmoc-Leu-OH by following the solid-phase peptide synthesis procedure. The crude material was then purified by reversed-phase chromatography (70 → 93% MeOH/H_2O + 5% of 2% formic acid in H_2O additive) to provide the desired product as a white solid (48% yield). HRMS *m/z* $[M+H]^+$ calcd for 1139.6604, found 1139.6631 (ES+).

Boc-Leu-Leu-Tyr-Leu-Leu-Leu-Tyr-Leu-OMe (4) was synthesized from Fmoc-Leu-OH by following the solid-phase peptide synthesis procedure. The crude material was then purified by flash chromatography (0 → 10% MeOH/DCM) to provide the desired product as a white solid (37% yield). HRMS *m/z* $[M+H]^+$ calcd for 1137.7175, found 1137.7195 (ES+).

Boc-Leu-Asp-Tyr-Tyr-Leu-OMe (7) was synthesized from Fmoc-Leu-OH by following the solid-phase peptide synthesis procedure. The crude material was then purified by reversed-phase chromatography (50 → 95% MeCN/H_2O) to provide the desired product as a white solid (31% yield). ^{1}H NMR (600 MHz, CD_3OD) δ 7.09 (d, *J* = 8.5 Hz, 2H), 6.91 (d, *J* = 8.0 Hz, 2H), 6.71 (d, *J* = 8.5 Hz, 2H), 6.65 (d, *J* = 8.4 Hz, 2H), 4.63 (t, *J* = 6.6 Hz, 1H), 4.50 (dd, *J* = 9.6, 5.2 Hz, 1H), 4.44 (dd, *J* = 9.4, 5.1 Hz, 1H), 4.35 (dd, *J* = 8.0, 4.9 Hz, 1H), 4.04 (dd, *J* = 9.6, 5.5 Hz, 1H), 3.68 (s, 3H), 3.10 (dd, *J* = 14.0, 5.2 Hz, 1H), 2.94 (dd, *J* = 14.3, 5.4 Hz, 1H), 2.90 – 2.81 (m, 2H), 2.80 – 2.68 (m, 2H), 1.71 – 1.56 (m, 4H), 1.44 (m, 11H), 0.94 (dd, *J* = 10.3, 6.4 Hz, 6H), 0.91 (d, *J* = 6.3 Hz, 6H); ^{13}C{^{1}H} NMR (151 MHz, CD_3OD) δ 175.9, 174.5, 174.1, 173.5, 173.3, 173.1, 158.2, 157.3, 157.2, 131.4, 131.1, 129.3, 128.8, 116.3, 116.3, 80.9, 57.2, 56.6, 54.8, 52.7, 52.2, 51.3, 41.8, 41.5, 37.6, 37.3, 36.2, 28.8, 25.9, 25.8, 23.4, 21.9; IR (FT-ATR, cm^{-1}, neat) ν_{max} 3292, 2958, 1645, 1514, 1440, 1367, 1228, 1159, 1019, 828; HRMS *m/z* $[M+H]^+$ calcd for $C_{40}H_{57}N_5O_{12}$ 800.4082, found 800.4091(ES+).

Boc-Leu-Asp-Tyr-Leu-Tyr-Leu-OMe (8) was synthesized from Fmoc-Leu-OH by following the solid-phase peptide synthesis procedure. The crude material was purified by reversed-phase chromatography (70 → 84% MeOH/H_2O + 5% of 2% formic acid in H_2O additive) to provide the desired product as a white solid (28% yield). HRMS *m/z* $[M+H]^+$ calcd for $C_{46}H_{68}N_6O_{13}$ 913.4922, found 913.4934 (ES+).

Boc-Leu-Asp-Tyr-Leu-Leu-Tyr-Leu-OMe (9) was synthesized from Fmoc-Leu-OH by following the solid-phase peptide synthesis procedure. The crude material was purified by reversed-phase chromatography (64 to 95% MeOH/H_2O + 5% of 2% formic acid in H_2O additive) to provide the desired

product as a white solid (20% yield). HRMS m/z $[M+H]^+$ calcd for $C_{52}H_{79}N_7O_{14}$ 1026.5763, found 1026.5771 (ES+).

Boc-Leu-Asp-Tyr-Leu-Leu-Leu-Leu-Tyr-Leu-OMe (10) was synthesized from Fmoc-Leu-OH by following the solid-phase peptide synthesis procedure. The crude material was purified by reversed-phase chromatography (64 → 95% $MeOH/H_2O$ + 5% of 2% formic acid in H_2O additive) to provide the desired product as a white solid (26% yield). HRMS *m/z* $[M+H]^+$ calcd for $C_{64}H_{101}N_9O_{16}$ 1252.7445, found 1252.7469 (ES+).

Procedure 4: Cu-catalyzed O-arylation of tyrosine-containing peptides. To an oven dried 50 mL Schlenk flask equipped with a magnetic stir bar was added tyrosine-containing substrate (0.02 mmol, 1.00 equiv), *N*-(2-bromophenyl)-2,2,2-trifluoroacetamide (0.0080 g, 0.03 mmol, 1.50 equiv), CuI (0.0019 g, 0.01 mmol, 0.50 equiv), ligand (0.0039 g, 0.02 mmol, 1.00 equiv), and Rb_2CO_3 (0.0460 g, 0.2 mmol, 10.00 equiv). The flask was sealed with a new rubber septum and further secured with Parafilm M®. The flask was placed under vacuum for 5 min and backfilled with N_2 x 3. Anhydrous, degassed THF (25 mL, 0.8 mM) was added through the septum, and the mixture was allowed to stir for 15 h at the 60 °C in an oil bath. After cooling to rt, the mixture was diluted with DCM and transferred to a separatory funnel. The organic layer washed with a solution of saturated NH_4Cl (aq) and the organic layer was separated. The aqueous layer was extracted with DCM x 3 and the combined organic layer was then dried with anhydrous Na_2SO_4, filtered, and concentrated in vacuo. The crude material was analyzed using LC-MS (see SI section 14 for determination of product distribution).

Procedure 5: Synthesis of authentic mono-proximal product. To confirm the major site of arylation, the authentic mono-proximal and distal products were prepared. Authentic standards of cross-coupled dityrosine containing peptides were synthesized following the general solution phase coupling procedure using **2-((tert-butoxycarbonyl)amino)-3-(4-(2-nitrophenoxy)phenyl)propanoic acid** as building.[21] After peptide coupling was performed, to a flask equipped with a magnetic stir bar was added intermediate methyl ((S)-2-((S)-2-((S)-4-(benzyloxy)-2-((S)-2-((tert-butoxycarbonyl)amino)-4-methylpentanamido)-4-oxobutanamido)-3-(4-(benzyloxy)phenyl)propanamido)-3-(4-(2-nitrophenoxy)phenyl)propanoyl)-L-leucinate (2.22 g, 2.25 mmol, 1.00 equiv) and 10 wt % Pd/C (0.22 g, 0.23 mmol, 0.10 equiv). The flask was purged with N_2 for 15 minutes, at which point MeOH was carefully added to the flask. The flask was equipped with a balloon of H_2, and was purged for 10 seconds. The flask was then left to stir at room temperature overnight. The mixture was filtered through Celite® and concentrated in vacuo to give a brown solid (1.28 g). This material was used in the next step without further purification. A small portion of the isolated material (0.56 g) was dissolved in DCM (6.3 mL, 0.1 M) and cooled to 0 °C while stirring. TFA_2O (0.098 mL, 0.69 mmol, 1.10 equiv) was then added dropwise to the solution. The solution was then allowed to warm to room temperature, and stirred for 15 min. The reaction was diluted with DCM, and washed three times with H2O. The organic layer was then concentrated in vacuo to give the crude residue, which was purified by reversed-phase chromatography (KP-C18-HS column, 20→100% $MeCN/H_2O$) to give the desired product **11b** as a white solid (0.018 g).

Boc-Leu-Asp-Tyr-Leu-Leu-Leu-Tyr(O-(2-(2,2,2-trifluoroacetamido)phenoxy)-Leu-OMe (5a) was synthesized following the Procedure 5. The crude material was purified by reversed-phase chromatography (75→100% $MeOH/H_2O$) to provide the desired product as a white solid (75% yield). HRMS m/z $[M+H]^+$ calcd for $C_{66}H_{94}F_3N_9O_{16}$ 1326.6849, found 1326.6907 (ES+).

Boc-Leu-Asp-Tyr-Tyr(O-(2-(2,2,2-trifluoroacetamido)phenoxy)-Leu-OMe (11a) was synthesized following Procedure 5. The crude material was then purified by reversed-phase chromatography (30→100% $MeOH/H_2O$) to provide the desired product as a white solid (11% yield). HRMS *m/z* $[M+H]^+$ calcd for $C_{48}H_{61}F_3N_6O_{13}$ 987.4327, found 987.4307 (ES+).

Boc-Leu-Asp-Tyr-Tyr(O-(2-(2,2,2-trifluoroacetamido)phenoxy)-Leu-OMe (11b) was synthesized following Procedure 5. The crude material was then purified by reversed-phase chromatography (20→100% $MeCN/H_2O$) to provide the desired product as a white solid (3% yield). HRMS *m/z* $[M+H]^+$ calcd for $C_{48}H_{61}F_3N_6O_{13}$ 987.4327, found 987.4305 (ES+).

Boc-Leu-Asp-Tyr(O-(2-(2,2,2-trifluoroacetamido)phenoxy)-Leu-Leu-Tyr-Leu-OMe (13a) was synthesized following Procedure 5. The crude material was then purified by reversed-phase chromatography (20 →100% $MeOH/H_2O$) to provide the desired product as a white solid (12% yield). HRMS *m/z* $[M+H]^+$ calcd for $C_{60}H_{83}F_3N_8O_{15}$ 1213.6008, found 1213.5990 (ES+).

Boc-Leu-Asp-Tyr-Leu-Leu-Tyr(O-(2-(2,2,2-trifluoroacetamido)phenoxy)-Leu-OMe (13b) was synthesized following Procedure 5. The crude material was then purified by reversed-phase chromatography (20→100% $MeOH/H_2O$) to provide the desired product as a white solid. HRMS *m/z* $[M+H]^+$ calcd for $C_{60}H_{83}F_3N_8O_{15}$ 1213.6003, found 1213.5990 (ES+).

Procedure 6: Synthesis of di-tyrosine containing hybrid peptide (15). Benzyl-protected precursor to di-tyrosine containing peptide hybrid **15** was synthesized following the solution phase coupling procedure using 7-((*tert*-butoxycarbonyl)amino)heptanoic acid[20] as building block. Global hydrogenolysis was performed after peptide coupling. To an oven-dried flask was added bis-benzyl ether (4.04 mmol, 1.0 equiv) and 2:1 MeOH/DCM (8 mL, 0.5 M). The reaction mixture was sparged with N_2 for 15 minutes. Pd/C (10 wt% - 50% wet with water, 0.60 g, 0.56 mmol, 0.14 equiv) was added to the mixture, and reaction mixture was sparged with N_2 for additional 15 minutes. Atmosphere was switched from N_2 to H_2 using a balloon of H_2. The reaction mixture was stirred at room temperature for 24 hours until hydrogenolysis of all three benzyl groups was observed (monitored by LC-MS). The mixture was filtered through Celite® and the solvent was

removed in vacuo to give a beige solid. The crude material was purified by reversed-phase chromatography

Procedure 6: Trifluoroacetylation of di-tyrosine containing hybrid peptide. To a round-bottomed flask equipped with a magnetic stir bar was added aniline bearing precursor to 16a or 16b (0.28 mmol, 1.0 equiv) and DCM (2.3 mL, 0.12 M). The resulting colorless solution was cooled to –78°C. Trifluoroacetic anhydride (TFA_2O) (54 μL, 0.39 mmol, 1.40 equiv) was added dropwise to the stirring mixture followed by dropwise addition of Et_3N (109 μL, 0.8 mmol, 2.80 equiv). The reaction mixture was stirred for 30 minutes until consumption of starting material was observed by TLC or LC-MS. The reaction mixture was removed from acetone bath and allowed come to room temperature and diluted with DCM. The reaction mixture was washed with 10% w/v citric acid. Aqueous layer was extracted with DCM x 3. Combined organic layers were washed with brine, dried over Na_2SO_4, filtered and concentrated in vacuo to yield beige amorphous solid.

(4S, 16S, 19S)-19-((tert-butoxycarbonyl)amino)-4,16-bis(4-hydroxybenzyl)-3,6,15,18-tetraoxo-2-oxa-5,14,17-triazahenicosan-21-oic acid (15) was synthesized following solution phase peptide coupling procedure followed by procedure 4. The crude material was purified by reversed-phase chromatography (0 → 40 → 90% MeCN/H_2O + 5% of 2% formic acid in H_2O additive) to provide the desired product as white solid (37% yield). ^{1}H NMR (600 MHz, CD_3OD) δ 7.02 (dd, *J* = 11.6, 8.2 Hz, 4H), 6.69 (d, *J* = 8.1 Hz, 4H), 4.61 (dd, *J* = 9.3, 5.5 Hz, 1H), 4.46 (t, *J* = 6.8 Hz, 1H), 4.36 (t, *J* = 6.7 Hz, 1H), 3.68 (s, 3H), 3.18–3.12 (m, 1H), 3.08–3.04 (m, 2H), 2.99–2.91 (m, 2H), 2.88–2.81 (m, 1H), 2.76 (dd, *J* = 16.9, 6.1 Hz, 1H), 2.61 (dd, *J* = 16.9, 7.2 Hz, 1H), 2.16 (t, *J* = 7.4 Hz, 2H), 1.50 (p, *J* = 7.5 Hz, 2H), 1.42–1.37 (s + m, 12H), 1.24–1.16 (m, 7H); ^{13}C{^{1}H} NMR (151 MHz, CD_3OD) δ 176.2, 174.2, 173.8, 173.4, 173.0, 157.8, 157.4, 157.4, 131.4, 131.2, 128.8, 128.6, 116.3, 116.2, 81.1, 56.2, 55.3, 52.8, 52.6, 40.5, 37.9, 37.6, 36.8, 36.6, 30.1, 30.1, 29.9, 28.7, 27.7, 26.8; IR (FT-ATR, cm-1, neat) ν_{max} 3345, 2933, 2957, 1645, 1612, 1514, 1226, 1259, 828, 536. HRMS m/z $[M+H]^+$ calcd for $C_{36}H_{50}N_4O_{11}$ 715.3554, found 715.3563 (ES+).

(4S, 16S, 19S)-19-((tert-butoxycarbonyl)amino)-4-(4-hydroxybenzyl)-3,6,15,18-tetraoxo-16-(4-(2-(2,2,2-trifluoroacetamido)phenoxy)benzyl)-2-oxa-5,14,17-triazahenicosan-21-oic acid (16a) was synthesized following the solution phase coupling procedure followed by Procedures 4, 5 using 7-((*tert*-butoxycarbonyl)amino)heptanoic acid and 2-(S)-2-amino-3-(4-(2-nitrophenoxy)phenyl)propanoic acid hydrochloride[21] as building blocks. Crude material was purified by (0 → 60 → 80% MeCN/H_2O + 5% of 2% formic acid additive) to provide desired product as a white solid (21% yield). ^{1}H NMR (600 MHz, CD_3OD): δ 7.64 (dd, *J* = 8.0, 1.6 Hz, 1H), 7.24 (dd, *J* = 7.9, 5.2 Hz, 3H), 7.16 (t, *J* = 7.9 Hz, 1H), 7.00 (d, *J* = 8.1 Hz, 2H), 6.92 (dd, *J* = 17.6, 8.3 Hz, 3H), 6.76 – 6.59 (m, 2H), 4.60 (dd, *J* = 9.3, 5.5 Hz, 1H), 4.55 – 4.49 (m, 1H), 4.38 (t, *J* = 6.7 Hz, 1H), 3.68 (s, 3H), 3.18 – 3.08 (m, 3H), 3.05 (dd, *J* = 14.0, 5.5 Hz, 1H), 2.96 (dd, *J* = 13.9, 8.3 Hz, 1H), 2.83 (dd, *J* = 13.9, 9.3 Hz, 1H), 2.75 (dd, *J* = 16.8, 6.3 Hz, 1H), 2.59 (dd, *J* = 16.7, 7.0 Hz, 1H), 2.14 (t, *J* = 7.4 Hz, 2H), 1.49 (p, *J* = 7.5 Hz, 2H), 1.41 (m, 11H), 1.25 – 1.17 (m, 6H); ^{13}C{^{1}H} NMR (151 MHz, CD_3OD) δ 176.2, 174.3, 173.8, 173.6, 172.9, 172.8, 157.7, 157.4, 157.3 (q, *J* = 37.5 Hz), 156.6, 152.1, 134.2, 131.9, 131.2, 129.3, 128.8, 127.3, 124.6, 120.0, 119.7, 117.5 (q, *J* = 287.2 Hz), 116.2, 81.0, 56.1, 55.3, 52.7, 52.6, 40.5, 38.0, 37.6, 37.0, 36.6, 30.2, 30.0, 29.9, 28.7, 27.7, 26.8; ^{19}F NMR (470 MHz, CD_3OD) δ –76.93; IR (FT-ATR, cm^{-1}, neat) ν_{max} 3291, 2934, 2859, 2494, 1718, 1643, 1541, 1456, 1156, 1022; HRMS *m/z* $[M+H]^+$ calcd for $C_{44}H_{54}F_3N_5O_{12}$ 902.3799, found 902.3806 (ES+).

(4S, 16S, 19S)-19-((tert-butoxycarbonyl)amino)-16-(4-hydroxybenzyl)-3,6,15,18-tetraoxo-4-(4-(2-(2,2,2-trifluoroacetamido)phenoxy)benzyl)-2-oxa-5,14,17-triazahenicosan-21-oic acid (16b) was synthesized following the solution phase peptide coupling procedure followed by Procedures 4 and 5 using 7-((*tert*-butoxycarbonyl)amino)heptanoic acid and methyl (*S*)-2-((tert-butoxycarbonyl)amino)-3-(4-(2-nitrophenoxy)phenyl)propanoate[21] as building blocks. The crude material was purified by reversed-phase chromatography (0 → 60 → 80% MeCN/H_2O + 5% of 2% formic acid additive) to provide desired product as a white solid (32% yield). ^{1}H NMR (600 MHz, CD_3OD) δ 7.64 (dd, J = 7.9, 1.8 Hz, 1H), 7.26 (ddt, J = 7.9, 6.4, 2.3 Hz, 1H), 7.22 – 7.14 (m, 3H), 7.02 (d, J = 8.1 Hz, 2H), 6.93 (dd, J = 8.6, 2.2 Hz, 3H), 6.70 (dd, J = 8.4, 1.7 Hz, 2H), 4.71 – 4.64 (m, 1H), 4.46 (t, J = 6.8 Hz, 1H), 4.37 (t, J = 6.7 Hz, 1H), 3.69 (s, 3H), 3.22 – 3.10 (m, 2H), 3.04 (dt, J = 13.1, 6.7 Hz, 1H), 2.99 – 2.89 (m, 3H), 2.77 (dd, J = 17.0, 6.1 Hz, 1H), 2.61 (dd, J = 16.9, 7.3 Hz, 1H), 2.23 – 2.07 (m, 2H), 1.52 (t, J = 7.2 Hz, 2H), 1.44 –1.37 (m, 11H), 1.30 – 1.07 (m, 6H); ^{13}C{^{1}H} NMR (151 MHz, CD_3OD) δ 176.2, 174.1, 173.5, 173.4, 173.0, 172.9, 157.8, 157.4, 157.3 (q, J = 37.4 Hz), 156.8, 151.9, 133.9, 131.7, 131.4, 129.3, 128.6, 127.4, 124.8, 120.0, 119.7, 117.4 (q, J = 287.3 Hz), 116.3, 81.1, 56.2, 55.1, 52.7, 40.5, 40.4, 37.9, 37.7, 36.7, 36.6, 30.1, 30.0, 29.9, 28.7, 27.6, 26.7; ^{19}F NMR: (376 MHz, CD_3OD) δ –76.93; IR (FT-ATR, cm^{-1}, neat) ν_{max} 3292, 2934, 2479, 1719, 1651, 1437, 1223, 1155, 1049, 760; HRMS m/z $[M+H]^+$ calcd for $C_{44}H_{54}F_3N_5O_{12}$ 902.3799, found 902.3801 (ES+).

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website. FAIR Data is available as Supporting Information for Publication and includes the primary NMR FID files for compounds: **1a-1d**, **2a-2d**, **3**, **4**, **5a**, **7-10**, **11a**, **11b**, **13a**, **13b**, **15**, **16a**, **16b**, **L25-L30**, **S2a-S2f**, **S3a-S3d**, **S4-S9**.

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Notes

The authors declare no competing financial interest.

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REFERENCES

1. (a) Liu, T. W. B.; Chen, J.; Zheng, G. Peptide-based molecular beacons for cancer imaging and therapy. Amino Acids, 2011, 41, 1123–1134; (b) Fosgerau, K.; Hoffmann, T. Peptide therapeutics: current status and future directions. Drug Discov. Today, 2015, 20, 122-128; (c) Nevola, L.; Giralt, E. Modulating protein–protein interactions: the potential of peptides. Chem. Comm., 2015, 51, 3302–3315.

2. (a) Li, H. Y.; Aneja, R.; Chaiken, I. Click chemistry in peptide-based drug design. Molecules, 2013, 18, 9797–9817; (b) Vlieghe, P.; Lisowski, V.; Martinez, J.; Khrestchatisky, M. Synthetic therapeutic peptides: science and market. Drug Discov. Today, 2010, 15, 40-56; (c) Wang, C.; Yang, C.; Chen, Y. C.; Ma, L.; Huang, K. Rational Design of Hybrid Peptides: A Novel Drug Design Approach. Curr. Med. Sci., 2019, 39, 349–355.

3. (a) Wang, W.; Lorion, M. M.; Shah, J.; Kapdi, A. R.; Ackermann, L. Late-Stage Peptide Diversification by Position-Selective C-H Activation. Angew. Chem. Int. Ed., 2018, 57, 14700–14717; (b) Zhu, Y. J.; Bauer, M.; Ploog, J.; Ackermann, L. Late-Stage Diversification of Peptides by Metal-Free C–H Arylation. Chem. Eur. J., 2014, 20, 13099–13102; (c) Boutureira, O.; Bernardes, G. J. L. Advances in Chemical Protein Modification. Chem. Rev., 2015, 115, 2174–2195.

4. (a) Ohata, J.; Martin, S. C.; Ball, Z. T. Metal-Mediated Functionalization of Natural Peptides and Proteins: Panning for Bioconjugation Gold. Angew. Chem. Int. Ed., 2019, 58, 6176–6199; (b) Zhang, C.; Vinogradova, E. V.; Spokoyny, A. M.; Buchwald, S. L.; Pentelute, B. L. Arylation Chemistry for Bioconjugation. Angew. Chem. Int. Ed., 2019, 58, 4810–4839; (c) Sletten, E. M.; Bertozzi, C. R. Bioorthogonal chemistry: fishing for selectivity in a sea of functionality. Angew. Chem. Int. Ed., 2009, 48, 6974–6998.

5. (a) Noisier, A. F. M.; Brimble, M. A. C–H Functionalization in the Synthesis of Amino Acids and Peptides. Chem. Rev., 2014, 114, 8775-8806; (b) Zhang, X.; Lu, G.; Sun, M.; Mahankali, M.; Ma, Y.; Zhang, M.; Hua, W.; Hu, Y.; Wang, Q.; Chen, J.; He, G.; Qi, X.; Shen, W.; Liu, P.; Chen, G. A general strategy for synthesis of cyclophane-braced peptide macrocycles via palladium-catalysed intramolecular sp^3 C–H arylation. Nat. Chem., 2018, 10, 540-548; (c) Li, B.; Li, X.; Han, B.; Chen, Z.; Zhang, X.; He, G.; Chen, G. Construction of Natural-Product-Like Cyclophane-Braced Peptide Macrocycles via sp^3 C–H Arylation. J. Am. Chem. Soc., 2019, 141, 9401–9407.

6. (a) Hoyt, E. A.; Cal, P.; Oliveira, B. L.; Bernardes, G. J. L. Contemporary approaches to site-selective protein modification. Nat. Rev. Chem., 2019, 3, 147-171; (b) Ohata, J.; Zeng, Y.; Segatori, L.; Ball, Z. T. A Naturally Encoded Dipeptide Handle for Bioorthogonal Chan-Lam Coupling. Angew. Chem. Int. Ed., 2018, 57, 4015-4019; (c) Zhang, C.; Welborn, M.; Zhu, T. Y.; Yang, N. J.; Santos, M. S.; Van Voorhis, T.; Pentelute, B. L. Π-Clamp-mediated cysteine conjugation. Nat. Chem., 2016, 8, 120-128.

7. (a) Ackermann, L. Carboxylate-Assisted Transition-Metal-Catalyzed C–H Bond Functionalizations: Mechanism and Scope. Chem. Rev., 2011, 111, 1315-1345; (b) Houpis, I. N.; Huang, C.; Nettekoven, U.; Chen, J. G.; Liu, R.; Canters, M. Carboxylate Directed Cross-Coupling Reactions in the Synthesis of Trisubstituted Benzoic Acids. Org. Lett., 2008, 10, 5601-5604; (c) Shi, B.-F.; Zhang, Y.-H.; Lam, J. K.; Wang, D.-H.; Yu, J.-Q. Pd(II)-Catalyzed Enantioselective C–H Olefination of Diphenylacetic Acids. J. Am. Chem. Soc., 2010, 132, 460-461.

8. (a) Cal, P.; Vicente, J. B.; Pires, E.; Coelho, A. V.; Veiros, L. F.; Cordeiro, C.; Gois, P. M. P. Iminoboronates: A New Strategy for Reversible Protein Modification. J. Am. Chem. Soc., 2012, 134, 10299-10305; (b) Chen, X.; Muthoosamy, K.; Pfisterer, A.; Neumann, B.; Weil, T. Site-Selective Lysine Modification of Native Proteins and Peptides via Kinetically Controlled Labeling. *Bioconjugate Chem.*, **2012**, *23*, 500-508; (c) Lee, H. G.; Lautrette, G.; Pentelute, B. L.; Buchwald, S. L. Pd-mediated Arylation of Lysine in Unprotected Peptides. *Angew. Chem. Int. Ed.*, **2017**, *56*, 3177-3181.

9. (a) Rojas, A. J.; Pentelute, B. L.; Buchwald, S. L. Water-Soluble Palladium Reagents for Cysteine S-Arylation under Ambient Aqueous Conditions. *Org. Lett.*, **2017**, *19*, 4263-4266; (b) Spokoyny, A. M.; Zou, Y. K.; Ling, J. J.; Yu, H. T.; Lin, Y. S.; Pentelute, B. L. A Perfluoroaryl-Cysteine SNAr Chemistry Approach to Unprotected Peptide Stapling. *J. Am. Chem. Soc.*, **2013**, *135*, 5946-5949; (c) Vinogradova, E. V.; Zhang, C.; Spokoyny, A. M.; Pentelute, B. L.; Buchwald, S. L. Organometallic palladium reagents for cysteine bioconjugation. *Nature*, **2015**, *526*, 687-691.

10. (a) Joshi, N. S.; Whitaker, L. R.; Francis, M. B. A Three-Component Mannich-Type Reaction for Selective Tyrosine Bioconjugation. *J. Am. Chem. Soc.*, **2004**, *126*, 15942-15943; (b) Tilley, S. D.; Francis, M. B. Tyrosine-Selective Protein Alkylation Using π-Allylpalladium Complexes. *J. Am. Chem. Soc.*, **2006**, *128*, 1080-1081; (c) Ichiishi, N.; Caldwell, J. P.; Lin, M.; Zhong, W.; Zhu, X.; Streckfuss, E.; Kim, H.-Y.; Parish, C. A.; Krska, S. W. Protecting group free radical C–H trifluoromethylation of peptides. *Chem. Sci.*, **2018**, *9*, 4168-4175.

11. a) Chinn, A. J.; Kim, B.; Kwon, Y.; Miller, S. J. Enantioselective Intermolecular C–O Bond Formation in the Desymmetrization of Diarylmethines Employing a Guanidinylated Peptide-Based Catalyst. *J. Am. Chem. Soc.*, **2017**, *139*, 18107-18114. b) Kim, B.; Chinn, A. J.; Fandrick, D. R.; Senanayake, C. H.; Singer, R. A.; Miller, S. J. Distal Stereocontrol Using Guanidinylated Peptides as Multifunctional Ligands: Desymmetrization of Diarylmethanes via Ullman Cross-Coupling. *J. Am. Chem. Soc.* **2016**, *138*, 7939-7945; c) Kwon, Y.; Chinn, A.; Kim, B.; Miller, S. J. Divergent Control of Point and Axial Stereogenicity: Catalytic Enantioselective C–N Bond-Forming Cross-Coupling and Catalyst-Controlled Atroposelective Cyclodehydration. *Angew. Chem. Int. Ed.* **2018**, *57*, 6251-6255.

12. Xie, X.; Chen, Y.; Ma, D. Enantioselective Arylation of 2-Methylacetoacetates Catalyzed by CuI/trans-4-Hydroxy-l-proline at Low Reaction Temperatures. *J. Am. Chem. Soc.,* **2006**, *128*, 16050-16051.

13. (a) Allen, C. L.; Leitch, D. C.; Anson, M. S.; Zajac, M. A. The power and accessibility of high-throughput methods for catalysis

research. *Nat. Catal.*, **2019**, *2*, 2-4; (b) Krska, S. W.; DiRocco, D. A.; Dreher, S. D.; Shevlin, M. The Evolution of Chemical High-Throughput Experimentation To Address Challenging Problems in Pharmaceutical Synthesis. *Acc. Chem. Res.*, **2017**, *50*, 2976-2985; (c) Mennen, S. M.; Alhambra, C.; Allen, C. L.; Barberis, M.; Berritt, S.; Brandt, T. A.; Campbell, A. D.; Castanon, J.; Cherney, A. H.; Christensen, M.; Damon, D. B.; de Diego, J. E.; Garcia-Cerrada, S.; Garcia-Losada, P.; Haro, R.; Janey, J.; Leitch, D. C.; Li, L.; Liu, F. F.; Lobben, P. C.; MacMillan, D. W. C.; Magano, J.; McInturff, E.; Monfette, S.; Post, R. J.; Schultz, D.; Sitter, B. J.; Stevens, J. M.; Strambeanu, J. I.; Twilton, J.; Wang, K.; Zajac, M. A. The Evolution of High-Throughput Experimentation in Pharmaceutical Development and Perspectives on the Future. *Org. Process Res. Dev.*, **2019**, *23*, 1213-1242.

14. Zhang, Y.; Yang, X.; Yao, Q.; Ma, D. CuI/DMPAO-Catalyzed *N*-Arylation of Acyclic Secondary Amines. *Org. Lett.*, **2012**, *14*, 3056-3059.

15. (a) Vagner, J.; Qu, H.; Hruby, V. J. Peptidomimetics, a synthetic tool of drug discovery. *Curr. Opin. Chem. Biol.*, **2008**, *12*, 292-296; (b) Fremaux, J.; Venin, C.; Mauran, L.; Zimmer, R. H.; Guichard, G.; Goudreau, S. R. Peptide-oligourea hybrids analogue of GLP-1 with improved action in vivo. *Nat. Commun.*, **2019**, *10*, 924; (c) Shu, J. Y.; Panganiban, B.; Xu, T. Peptide-Polymer Conjugates: From Fundamental Science to Application. *Annu. Rev. Phys. Chem.*, **2013**, *64*, 631-657.

16. Collins, K. D.; Glorius, F. Intermolecular Reaction Screening as a Tool for Reaction Evaluation. *Acc. Chem. Res.* **2015**, *48*, 619–627

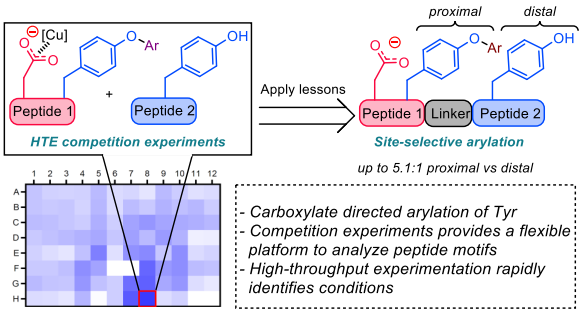
17. Fulmer, G. R.; Miller, A. J. M.; Sherden, N. H.; Gottlieb, H. E.; Nudelman, A.; Stoltz, B. M.; Bercaw, J. E.; Goldberg, K. I. NMR Chemical Shifts of Trace Impurities: Common Laboratory Solvents, Organics, and Gases in Deuterated Solvents Relevant to the Organometallic. *Organometallics* **2010**, *29*, 2176-2179.

18. Zhang, Y.; Yang, X.; Yao, Q.; Ma, D. CuI/DMPAO-Catalyzed *N*-Arylation of Acyclic Secondary Amines. *Org. Lett.* **2012**, *14*, 3056-3059.

19. Romney, D.K.; Colvin, S. M.; Miller, S.J. Catalyst Control over Regio- and Enantioselectivity in Baeyer–Villiger Oxidations of Functionalized Ketones. *J. Am. Chem. Soc.* **2014**, *136*, 14019-14022.

20. Gavande, N.; Kim, H.; Doddareddy, M. R.; Johnston, G. A. R.; Chebib, M.; Hanrahan, J.R. Design, Synthesis, and Pharmacological Evaluation of Fluorescent and Biotinylated Antagonists of ρ 1 GABAC Receptors. *ACS Med. Chem. Lett.* **2013**, *4*, 402–407.

21. Beugelmans, R.; Bigot, A.; Zhu, J. A new access to 14-membered macrocycle: Synthesis of model F-O-G ring of teicoplanin. *Tetrahedron Lett.* **1994**, *35*, 5649–5652.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Peptide 1
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HTE competition experiments
Apply lessons
proximal
distal
Peptide 1
Linker
Peptide 2
Site-selective arylation
up to 5.1:1 proximal vs distal
- Carboxylate directed arylation of Tyr
- Competition experiments provides a flexible platform to analyze peptide motifs
- High-throughput experimentation rapidly identifies conditions